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RESEARCH ARTICLE

Morphological changes of gonad and gene expression patterns during desexualization in *Dugesia japonica* (Platyhelminthes: Dugesiidae)

Zimei Dong[1], Changying Shi[1], Gengbo Chu[1], Yanping Dong[1], Guangwen Chen[1], Dezeng Liu[1]

[1]*College of Life Science, Henan Normal University. Xinxiang, 453007 Henan, China.*
Corresponding author: Guangwen Chen (chengw0183@sina.com)

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ABSTRACT. Planarians, the representatives of an ancient bilaterian group with complex reproductive system and high regenerative capabilities, are model system suitable for studying the basic molecular requirements for the development of the reproductive system. To further explore the morphological changes of the gonads during desexualization and the molecular events of the genes controlling the reproductive system development in planarians, we have investigated the histological changes of ovary and testis by paraffin section and the expression patterns of reproductive-related genes by the quantitative real-time PCR in *Dugesia japonica* Ichikawa & Kawakatsu, 1964, upon starvation. The four genes, *Djprps*, *DjvlgA*, *DjvlgB* and *Djnos*, have been selected. The research results show that the degradation of ovary changes from outside layer to inside, and the testis changes are opposite; the reproductive capacity of the planarians starts to be damaged from the 17th to 25th days and to disappear completely from the 26th to 37th days during starvation. The expression patterns of the four genes exhibit the obvious dynamic variations during their desexualization, which indicates that these genes might be involved in gonad development.

KEY WORDS. Planarian, food deprivation, germ cells, reproductive system.

INTRODUCTION

Organisms have their definite and unique reproductive system to propagate and maintain their species, and asexual and sexual reproductions, which are two types of reproductive mode, have been formed in the process of biological evolution (Bell 1982). Platyhelminthes have often been regarded as resembling the ancestor of all bilateria (Moore and Willmer 1997). Planarians are the earliest free-living Platyhelminthes with triploblastic and bilateral symmetry, which play an important role in the evolution of the metazoan system. Planarian *Dugesia japonica* Ichikawa & Kawakatsu, 1964, with hermaphroditic reproductive organs, has both asexual (by fission) and sexual (by oviparity) reproduction, and the two reproduction modes can be interconvertible due to some factors, including seasons, temperature and food (Kenk 1940, Hase et al. 2007).

The reproductive system of *D. japonica* consists of the male and female gonads as well as accessory reproductive organs (Newmark et al. 2008). Numerous testes distribute into two longitudinal bands in the dorso-laterally space, and a pair of ovaries are situated ventrally at the posterior region of the brain. The ciliated oviducts and the sperm ducts running along the nerve cords lead into the copulatory apparatus, which is comprised of the genital pore, seminal vesicles, copulatory bursa, bursal canal, penis and various glands. In each planarian testis lobe, spermatogenesis proceeds from the periphery to the lumen. Spermatogonia undergo three mitotic divisions with incomplete cytokinesis to produce eight primary spermatocytes that enter meiosis, differentiate into 32 spermatids, and mature into spermatozoa (Franquinet and Lender 1973). The sperms are released into the sperm ducts that funnel sperms to the seminal vesicles. When *D. japonica* mates, the sperms from one worm are transferred to the partner and are deposited via the bursal canal into the copulatory bursa. Then the sperms travel back down the bursal canal into the oviducts and are collected in the tuba, an enlarged portion of the oviducts just outside the ovaries (Chong et al. 2011). As the mature oocytes leave the ovary, they are fertilized by the sperms stored in the tuba. The fertilized eggs then make their way down the oviduct, and the yolk cells are added to the outside of the eggs by the vitelline glands that line the oviduct. Several embryos and yolk cells are packaged into a single egg capsule. The glands around the genital atrium are involved in the synthesis and deposition of the egg capsules (Shinn 1993). Therefore, the germ cells from the testis and the

ovary play critical roles in the development and maintenance of reproductive system in planarians.

The existence of the two divergent modes of reproduction in a single species presents a unique opportunity to study reproductive-related genes. Up to now, a large number of reproductive-related genes have been isolated in planarians, such as *DjFGFR2*, *vasa-like*, *DeY1*, *Dryg*, *nanos*, *piwi* and *DjPRPS*. These genes are expressed in the reproductive system of sexual worms and are involved in the formation of reproductive system (Ogawa et al. 1998, Shibata et al. 1999, Salvetti et al. 2002, Hase et al. 2003, Nakagawa et al. 2012, Rouhana et al. 2014, Shi et al. 2015). However, the mechanisms of the germ cell development and reproductive maturation of planarians remain largely unknown.

This study has investigated the morphological changes of the ovary and testis during the reproductive system degeneration induced by starvation in *D. japonica*, and simultaneously, analyzed the dynamic expression patterns of the four genes (*Djprps*, *DjvlgA*, *DjvlgB* and *Djnos*) by the real-time PCR. This research has provided the basic data to explore the mechanism of planarian reproduction.

MATERIAL AND METHODS

The planarians used in this study belong to the sexual strain of the species *D. japonica*, collected from Yuquan Spring, Henan Province, China. Animals were fed beef liver once every six days and cultured in autoclaved tap water in dark at 22 °C. To induce the sexual worms desexualization, they were starved for 6 (the control), 16, 25, 37 and 44 days, respectively. The date when they were last fed was regarded as the 0 day after starvation.

Paraffin section of the gonads was performed as described previously (Dong et al. 2011). Briefly, starved worms were fixed in Bouin's fluid, followed by rinses with 70% ethanol, dehydrated in an ascending series of ethanol solutions, cleared in xylene, and embedded in synthetic paraffin. Serial sections were made at intervals of 8 μm and were stained with hematoxylin and eosin. Images were acquired with a Leica digital camera (DP70; Olympus) attached to a compound microscope (BX40; Olympus, Hamburg, Germany).

Quantitative real-time PCR was carried out as described previously (Dong et al. 2012). The *Djβ-actin* gene, a fragment of 121 bp, was used as the internal control (accession number: AB292462). The expression levels on the day 6th after the last feeding were used as the control, all primers (Table 1) were designed using Oligo 6.0 software, and all primers generated a single PCR band of the expected size. PCR products were verified by DNA sequencing.

Table 1. Genes and their primer sequences used for real-time PCR analysis.

Genes	Primers	Sequences(5′–3′)
Djprps	DjPrps F	TCAGGCGTCTATGTGATTGCTACT
	DjPrps R	AGACCGATTCGCCGTAATGTAT
DjvlgA	DjvlgA F	GTTAGTGCGTCCTTGCGTGGT
	DjvlgA R	GTTGTCCAGGAGGCGGCAT
DjvlgB	DjvlgB F	CTACATTCAATAGACAACAGGCTCC
	DjvlgB R	TTGTTGGTGATGGATGGGTTC
Djnos	Djnos F	GGAAGATGACCACGACAAC
	Djnos R	GCTTCGGTCCAGTGTTATGT

F: forward primer; R: reverse primer.

RESULTS

Histological changes of the ovary during starvation

For sexual maturity individuals, the outer layer of the ovary includes a lot of round, small, basophilic cells, which are oogoniums. Many larger oocytes are clearly visible within the ovary, and their nucleus deviate from the central part of the cell (Fig. 1, 0d). When the worms are starved for six days, the ovary remains almost unchanged compared with that of 0 day. On the 16th day after starvation, the size of the ovary gets small, and the number of the oogoniums begins to decrease. The nucleus of some oocyte is invisible on the 25th day after starvation, the number of oogonium decreases rapidly, and the membrane of oocyte is disintegrated, and the cell boundary is not clear. On the 37th day after starvation, the oogoniums almost fully disappear in the ovaries, the nucleus of most oocytes is dissolute, and the cell boundary is not clear. Until the 44th day after starvation, the oogoniums disappear completely, the oocytes continue to decrease in number, and the cell boundary is blurred. The ovaries grow smaller in volume during starvation (Fig. 1).

Histological changes of the testis during starvation

The testis can be obviously divided into two layers: the larger spermatogoniums and spermatocytes are in the outer layer, while the spermatids and spermatozoa, spindle and basophilic, occupy the inner region (Fig. 2, 0d). There are no significant changes in testis on the 6th day after starvation. On the 16th day after starvation, the number of the spermatids and the spermatozoa is gradually reduced. On the 25th day, the number of the spermatids and the sperms decrease dramatically, and at the same time, the spermatocyte also decreases in quantity. After 37 days' starvation, only a few sperms are observed. On the 44th day after starvation, the mature sperms disappear completely in the testis, and the boundary between the spermatogoniums is not clear. Similar to the ovaries, the testes also become smaller in volume during starvation (Fig. 2).

The expression level of the reproductive-related genes during starvation

There is no difference in the morphology and histology of the gonads on the 6th day after starvation compared with those of 0 day. In order to avoid the influence of the undigested food,

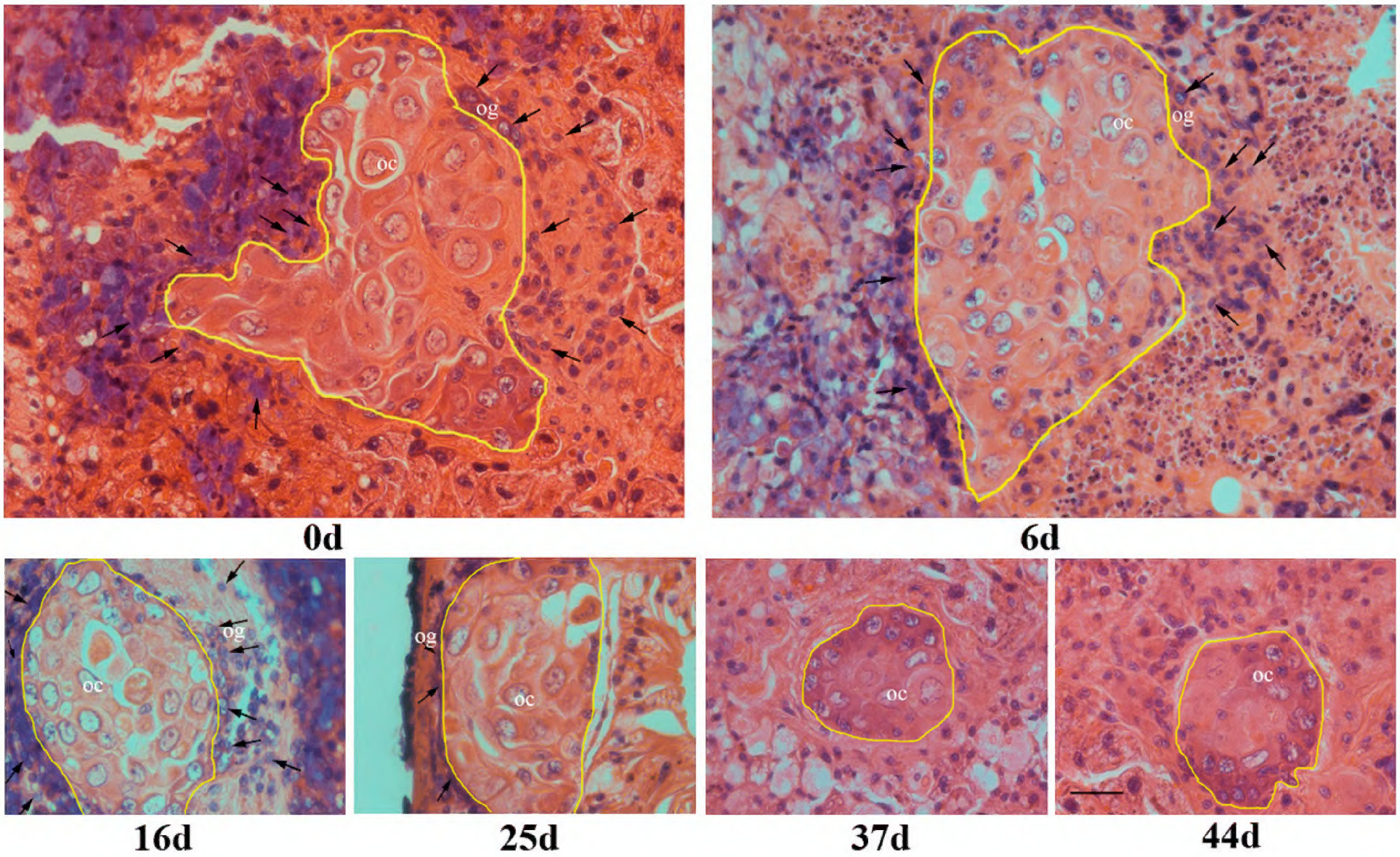


Figure 1. Histological changes of the ovary during starvation. The ovary of on the day 25th is sagittal section and the others are horizontal section. The yellow lines indicate the innermost region and the oogoniums marked by black arrows, d: days after starvation, og: oogonium, oc: oocyte. Scale bar: 200 μm.

the expression levels of the genes on the 6th day after food deprivation are used as the control. This study has adopted four reproductive-related genes, *Djprps*, *DjvlgA*, *DjvlgB* and *Djnos*, and has observed their expression levels during the gonads degradation.

The research results show that the four genes have the similar expression patterns. Their expression levels reduce gradually from the 6th day, reach the lowest levels on the 25th day, and then their expression levels increase. The expression levels of *Djprps* and *Djnos* reach the peak on the 44th day after starvation (Figs 3, 6), but the expression levels of *DjvlgA* and *DjvlgB* reach the peak on the 37th day after starvation (Figs 4, 5).

DISCUSSION

Freshwater planarians exhibit strong tolerance to prolonged starvation. An adult worm can still survive after several months of starvation, but the reproductive system of the worm disappears gradually in the process of starvation due to maintaining the individual normal function, that is, the desexualization is induced by starvation. The significant changes of the gonads are observed during the desexualization, which are divided into three stages. On the first stage, from the 0 to 16th days, the ovaries and testes grow smaller, and the oogoniums, spermatid and spermatozoa begin to reduce in number. On the second stage, from the 17th to 25th days, the number of the mature sperms and oogoniums decreases dramatically, and some oocytes are damaged. On the third stage, from the 26th to 44th days, the oogoniums and the mature sperms continue to decrease until they disappear completely, and most oocytes are damaged and the boundary of the spermatogoniums is not clear. What is more, the different changes of the ovary vary from the testis during desexualization, and the oogoniums start to degenerate until they disappear in the ovary. Instead, for the testis, the mature sperms start to decrease, but the spermatogoniums can be kept for a longer time. This is a very interesting phenomenon, although the mechanism is unknown. Based on the comprehensive analysis, this study draws a hypothesis conclusion that the reproductive capacity of planarian damages on the second stage and disappears completely on the third stage.

In order to explore the expression patterns of the reproductive-related genes during starvation, this study focuses on the four genes involved in reproductive system development and monitors their expressions by quantitative real-time PCR. Their expression levels reduce gradually from the 6th day and

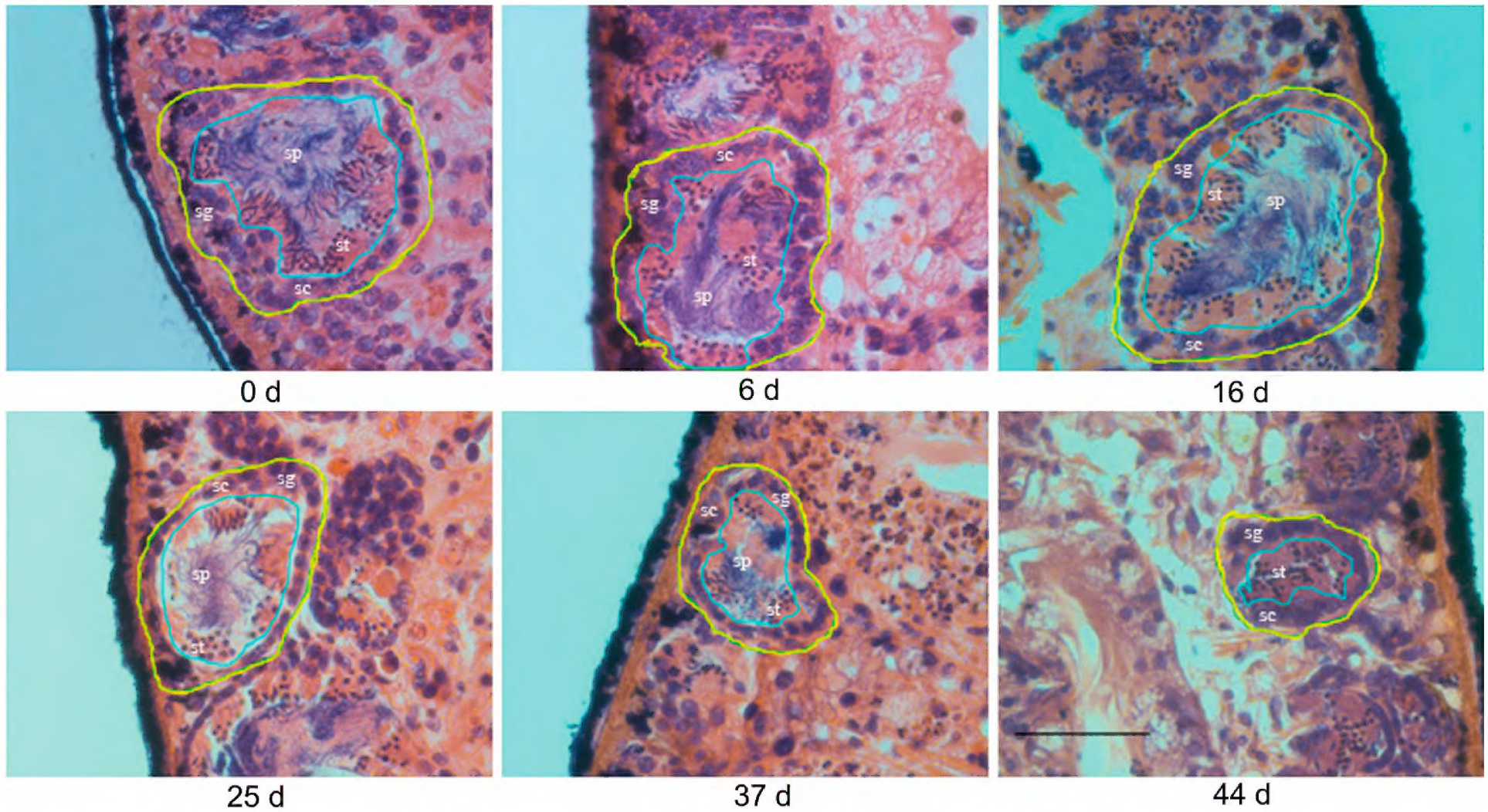
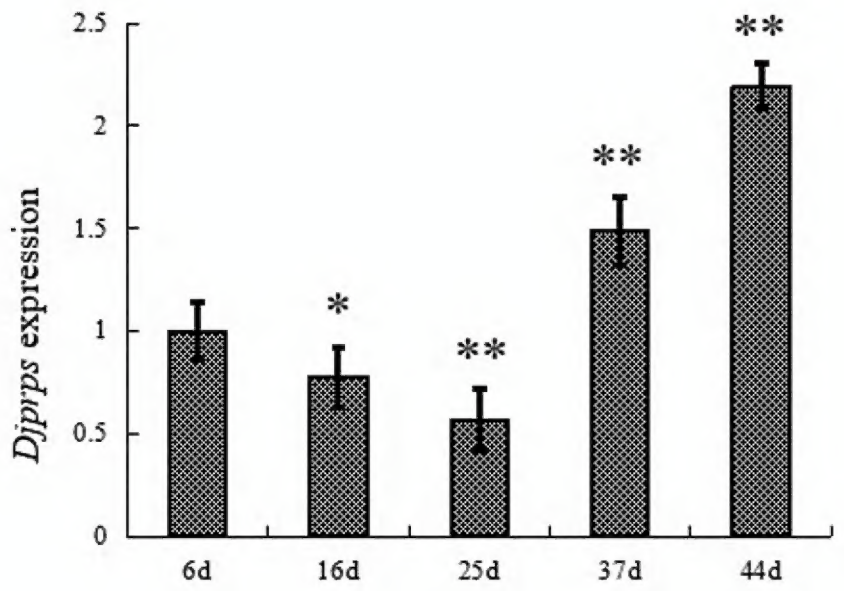


Figure 2. Sagittal section of testis during starvation. The blue lines indicate the innermost region and the yellow lines indicate that the border between the testis region and the surrounding tissue. d: days after starvation, sg: spermatogonium, sc: spermatocyte, st: spermatid, sp: spermatozoa. Scale bar: 100 μm.

reach the lowest levels on the 25th day, which is the critical stage when the reproductive capacity of planarian is damaged. As a result, we think these genes play important roles during the gonad development. However, their transcripts are up-regulated unexpectedly on the 37th or 44th day after starvation. Meanwhile the gonads continue their degradation on this stage. The precise function of each gene in the gonad remains unknown, and needs to be worked out in the future research of the planarians.

The *prps* gene has been cloned from planarians *Dugesia. Ryukyuensis* Kawakatsu, 1976 and *D. japonica*, respectively. Its spatial expression is in bilateral lines of patches in the ventral side where are located the ovary and the yolk glands of sexual worms, which indicates that *prps* might be one of sexual reproduction related genes in planarians (Hase et al. 2007, Shi et al. 2015). Ribose-phosphate pyrophosphate kinase (PRPS) is an important enzyme and is responsible for the synthesis of 5-phosphoribosyl-1-pyrophosphate (PRPP), a precursor required for the production of purine, pyrimidine and pyridine nucleotides and histidine and tryptophan (Sakakibara 1992). PRPS is required for both the de novo and salvage pathways and therefore, represents a key enzyme in intermediary metabolism (Carter et al. 1997). In planarians, a pair of ovaries are firstly developed during sexualization (Kobayashi and Hoshi 2002, 2011), and ovarian maturation is important for the development of the other reproductive organs (Maezawa et al. 2014). In this study, *Djprps* expression is gradually reduced from the 6th to 25th days after starvation, in which the oogoniums decrease greatly. However, with the disappearance of the oogoniums and the disintegration of oocyte membrane, *Djprps* expression levels begin to rise and reach the peak. These data suggest that *Djprps* might exist in the oogoniums and is a key regulator of the oogoniums development. Meanwhile, it might be involved in metabolism on the later stage, when the oogoniums and most oocytes are damaged, or it is perhaps a compensatory response for the decrease of the germ cells.

Vasa, a member of the ATP-dependent DEAD-box RNA helicase family, is a prototypic marker of the primordial germ cells and of the germ line in the metazoan (Gustafson and Wessel 2010, Skinner et al. 2012, Diao et al. 2015). Vasa-like homologues have been reported in Platyhelminthes including *Macrostomum lignano* Ladurner, Schärer, Salvenmoser & Rieger, 2005, *Schmidtea polychroa* (Schmidt, 1861), *Neobenedenia girellae* (Hargis, 1955), *Paragonimus westermani* Kerbert, 1878 and several *Dugesia* species (Shibata et al. 1999, Mochizuki et al. 2001, Ishizuka et al. 2007, Ohashi et al. 2007, Pfister et al. 2008, Solana et al. 2009). Two vas-related genes, *DjvlgA* and *DjvlgB* are isolated from the planarian *D. japonica*. The transcripts of both *DjvlgA* and *DjvlgB* are detected in the oocytes. However, the expression patterns of *DjvlgA* and *DjvlgB* are distinguishable in testis. *DjvlgA* is expressed in spermatogonia, spermatocyte, and spermatid,



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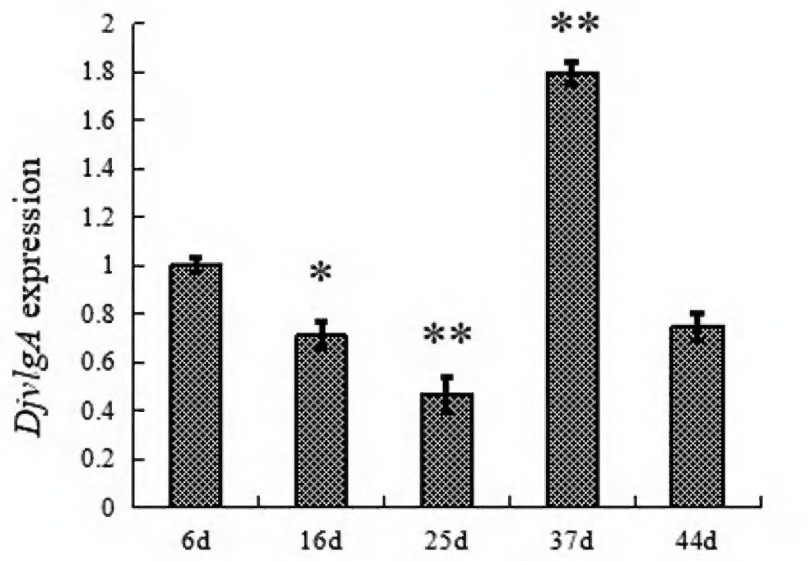

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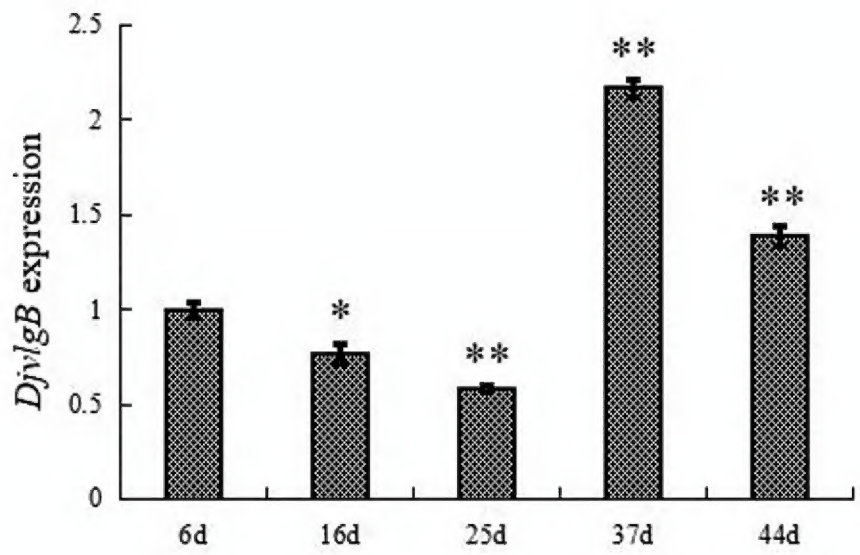

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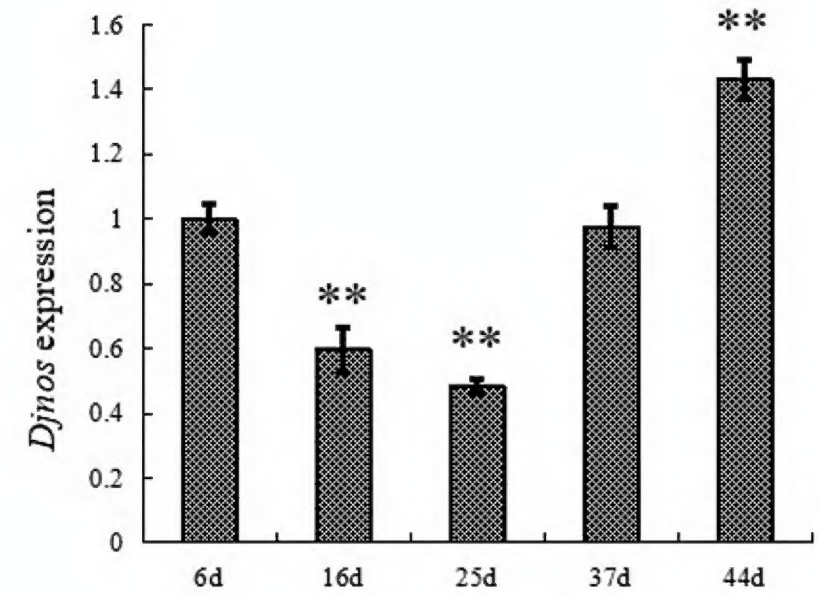

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Figures 3–6. The time-course expressions of four genes during starvation by real-time PCR: (3) *Djprps*; (4) *DjvlgA*; (5) *DjvlgB*; (6) *Djnos*. The expression levels on the day 6th after the last feeding were used as the control, *β-actin* was used as inter-control. d: days after starvation. Vertical bars represented the mean ± SD (N = 3). Asterisks indicate statistical differences (*p < 0.05, **p < 0.01).

but not in mature sperm. Moreover, *DjvlgA* is also expressed in a number of somatic stem cells in the mesenchymal and may play an important role in differentiation and maintenance of the cell state of the germ cells as well as the somatic stem cells. The expression of *DjvlgB*, in contrast to that of *DjvlgA*, is restricted to spermatocyte (Shibata et al. 1999). In this study, both *DjvlgA* and *DjvlgB* have the similar expression patterns, and their transcripts decrease gradually from the 6 th to 25th days. We speculate that the two genes might be involved in the germ cells differentiation and the gonad function maintenance. But their transcripts are up-regulation again on the 37 th day, so we believe that they also participate in germ cells metabolism on the later stage in the process of desexualization. At the same time, the expression levels of *DjvlgA* on the 44th day are different from those of *DjvlgB*, which may result from the reduction of the somatic stem cells in number.

The *nanos* gene encodes an RNA-binding protein containing a zinc finger motif, which is conserved in diverse metazoan (Wang et al. 2010). Homologs of *nanos* are required for the formation and maintenance of germ line stem cell systems and for gametogenesis in many metazoans (Nakagawa et al. 2012). *Djnos* is first cloned from *D. japonica* and expressed in ovary or testis in sexual worms, and it distributes to the prospective ovary or testis forming region in the sexual worms (Sato et al. 2006). In addition, the *Djnos*-expressed cells produce the germ cells and then differentiate into the oogoniums and spermatogonia during sexualization (Wang et al. 2010). In this study, the expression patterns of *Djnos* reveal the similarity to those of *Djprps*. These data prove that the function of *Djnos* is similar to that of *Djprps*, which is involved in the early gonad development and is related to the metabolism of germ cells at the later stage.

Of course, it is important to note that these genes might

be pleiotropic, which means that they also serve an apparently different function elsewhere apart from their role in gonad formation (Solana et al. 2009, Rouhana et al. 2014). Therefore, the functional analysis of the related genes should be combined with the previous reports. *DjvlgA* and *DjvlgB*, for example, might take part in the whole process of gonad development and have different functions at the different stages. *Djnos* might act on the other function of gonadogenesis.

In conclusion, this study finds out the histological changes of the testis and the ovary and the different expression patterns of reproductive-related genes during desexualization by starvation. The results of this research suggest that the degradation of the ovary is from the outside layer to the inside whereas the testis is opposite. Gonad degradation results from the polygenic services and the reproductive-related genes might play important roles during desexualization. In order to better understand the molecular basis of the planarian gonad degradation, it might be of great use and significance to further analyze the interactions of these genes in the network apart from the function of every gene during desexualization.

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